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In this report, proliferating and differentiated myogenic cells have been used to evaluate the effects of hypoxia on the cell cycle distribution and protein synthesis in vascular smooth muscle. The data demonstrate potentiation of myocyte responsiveness to growth stimuli by hypoxia. The modulation of phenotype was also observed as reduced glucose consumption and lactate production rates. Although the induction of GRPs and ORPs in myogenic cells was independent of their state of differentiation; suggesting that these are generic responses to ischemic stress; differences in protein synthesis were observed. Hypoxia generally induced or enhanced protein synthesis in myocytes but primarily inhibited protein synthesis in myoblasts. Moreover, two proteins (PSP100 and PSP9) were identified among those induced in myocytes by hypoxia, which were otherwise characteristic of the myoblast phenotype. Taken together, the data suggest that metabolic stresses, such as hypoxia, can modulate the differentiated cell phenotype with proliferative characteristic.

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PRINCIPAL INVESTIGATOR: Harold C. Smith, Ph.D.

CONTRACTOR: University of Rochester
Department of Pathology

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INTRODUCTION

Dense preparations of primary-culture, quiescent, vascular smooth-muscle cells are morphologically and functionally similar to densely packed smooth muscle cells in situ in that they can be induced to contract in response to chemical and electrical stimulation (1,2). When sparsely seeded, the cells lose their contractile characteristic within one week and once so "modulated", acquire proliferative responsiveness to serum mitogens. Another characteristic of modulated myocytes is that they no longer express smooth muscle specific alpha-actin and myosin (3-6). The transition between quiescent and proliferative phenotypes has also been observed in vivo following experimental in / of blood vessels from immature rats and during atherogenesis in humans (1,2,7-14). Hypoxia has been suggested specifically, as being responsible for the phenotypic modulation response of vascular smooth muscle cells in vivo (8,9) and in vitro (10), and to induce modulation of the proteins synthesized and secreted by vascular smooth-muscle cells (8,10).

The molecular mechanisms for vascular smooth-muscle phenotypic modulation are still unknown. At least part of the answer appears to be related to the finding that confluent, quiescent smooth muscle cells and endothelial cells in vivo (15,16) and in vitro (17,18) produce a heparin sulfate glycosaminoglycan as an extracellular matrix component of their basement membranes. Heparin sulfate isolated from basement membrane preparations has been shown to inhibit smooth muscle cell proliferation and stimulate secretion of several different types of proteins (11-13). It has been proposed that these myocyte secretions are autocrine growth regulatory factors which promote differentiation (2,8,10,11,14). The effect of hypoxia on the growth factor composition of the extracellular matrix and on the interactions(s) of myocytes with these, and other soluble growth factors is unknown. In a somewhat different context, tumor microenvironments are known to play a major role in regulating cell proliferation and in influencing the efficacy of radiation and chemotherapy (19,20). Deficiencies in tumor microvasculature establish ischemic microenvironments which lead to heterogeneity in tumor cell growth characteristics and responsiveness to therapy (3,19-28).

A predominant metabolic response of cells to hypoxia is an increased rate of glucose consumption (Pasteur effect) with a corresponding decrease in oxygen consumption. It is generally believed that the effect arises from a shift in oxidative

phosphorylation to the glycolytic pathway for energy production. Measurements of microenvironmental oxygen within blood vessels of tumors or within spheroids (using oxygen microelectrodes) or of oxyhemoglobulin saturation in capillaries surrounding and within tumors (using cryospectrophotometry) have recorded oxygen levels $\leq$ 10 mm (19,20). In fact, lactate production and acidification of the microenvironment are important indicators of the shift in the metabolic pathways. Changes in either can modulate tumor cell growth and metabolism even in the presence of optimal oxygen and glucose concentrations (19,20).

In addition to metabolic alterations, hypoxia has been shown to alter gene expression. Hypoxia has been demonstrated to induce aberrant DNA replication patterns (29,30) and glucose-starvation and/or hypoxia can lead to a depletion of nuclear topoisomerase II (31). Messenger RNA levels for some of the glycolytic enzymes are enhanced by hypoxia (32). Several proteins whose functions are presently not known have also been reported to have enhanced rates of synthesis during hypoxia (33-36) and glucose starvation (37-40). In mammalian cells the two major glucose-regulated proteins (GRPs) and three major oxygen-regulated proteins (ORPs) have molecular weights of 97 and 78 and 260, 150 and 33 respectively. An intriguing possibility is that GRPs and ORPs are part of a general adaptive mechanism cells have evolved, to cope with metabolic stresses.

ORPs have been identified by polyacrylamide gel analysis at varying constitutive levels in many aerobic rodent and human cells (19,33,34,36). Enhanced synthesis of ORPs was observed as early as 1 hour following establishment of hypoxia, and reached maximum levels by 10-14 hours of hypoxia. The enhancement appears to be due to differential translation and transcription rates (33,36). ORPs can be induced by hypoxia in the presence of normal glucose levels (5.5 mM) but low glucose (0.4 mM) in well oxygenated cultures can induce 78 and 98 kDa GRPs which have been suggested to be the same as the ORPs 80 and 100 kDa (19,22,24,25,37). Direct peptide sequencing confirmed that ORP80 and GRP78 are identical[1]. The potential role of low glucose in the induction of ORP 260, 150 and 33 has also been suggested (37).

In this manuscript, proliferating and differentiated myogenic cells have been used to evaluate the effects of hypoxia on cell cycle distribution and protein synthesis in vascular smooth muscle. The data demonstrate potentiation of myocyte responsiveness to growth stimuli by hypoxia. The modulation of phenotype was also observed as reduced glucose consumption and lactate production rates. Although the induction of GRPs and ORPs in myogenic cells was independent of their state of differentiation, suggesting that these are generic responses to ischemic stress; differences in protein synthesis were observed. Hypoxia generally induced or enhanced protein synthesis in myocytes but primarily inhibited protein synthesis in myoblasts. Moreover, two proteins (PSP100 and PSP9) were identified among those induced in myocytes by hypoxia, which were otherwise characteristic of the myoblast phenotype. Taken together, the data suggest that metabolic stresses, such as hypoxia, can modulate the differentiated cell phenotype with proliferative characteristic.

RESULTS

Phenotypic characteristics of differentiated BC_3H1 myogenic cells. BC_3H1 cells were originally cloned from a rhabdomyosarcoma derived from mouse brain (41) and have since been characterized morphologically and biochemically as a smooth muscle-like cell line (4,5) or alternatively as a non-terminally differentiated skeletal muscle cell line (42). The BC_3H1 cell line grows readily (doubling time 30 hr) and can be easily induced to differentiate by contact inhibition and serum-free media. These characteristics together with

the smooth muscle-like morphology and biochemical properties of differentiated cultures make BC_3H1 cells a useful in vitro model for evaluating the response of the microvasculature to ischemic stress.

Proliferating cells (myoblasts) grown in the presence of 10% fetal calf serum have a highly flatten appearance and make numerous contacts with neighboring cells through cytoplasmic projections (Figure 1A). DNA histograms of mid log phase cultures revealed a cell cycle distribution consisting of 40% in G_0/G_1 phases, 35% in S phase and 25% in G_2/M phases (Figure 2A).

Cells accumulated in G_0/G_1 upon establishing a confluent monolayer (Figure 2C); demonstrating that cell growth was contact inhibited. Differentiation was induced as described in Materials and Methods by culturing confluent monolayers in serum-free defined-media for 4-5 days. During this time, cell growth remained contact inhibited (Figure 2E) and the cell morphology progressed towards large arrays of highly elongated non-fusing syncytia of cells typical of the appearance of smooth-muscle (Figure 1B). Differentiated BC_3H1 cells (myocytes) also secret and condition an extracellular matrix containing glycosaminoglycans with autocrine factors typical of smooth muscle (15,16,18,21-29).

Coincident with growth and morphological alterations, the glucose consumption rate progressively increased as cells differentiated (Table I). Myocytes had twice the glucose consumption rate of myoblasts. Lactate production also markedly increased and reached greater than 3-fold of the myoblast level in fully differentiated cells (Table I).

The effect of hypoxia on cell survival. Cells were made hypoxic as described in Materials and Methods for varying lengths of time and subsequently plated at various dilutions in order to determine cell survival following hypoxia. Myoblasts and myocytes had similar levels of cell survival from 7-22 hours of hypoxia but differed in their initial and long-term responses (Figure 3). Myoblasts (mid log phase) were initially more resistance to hypoxia (the shoulder on the survival curve in Figure 3) but following 3 hours, demonstrated a progressive decrease in clonicity with increasing duration of hypoxia. No growth could be recovered from cultures which had been hypoxic for $\geq$ 25 hours. Myocytes demonstrated a marked sensitivity to short durations of hypoxia but had a similar survival as myoblasts from hypoxia lasting 7-22 hours. Myocytes were less sensitive to long term hypoxia than proliferating cells (Figure 3). Myocytes were no longer clonable following 48 hours of hypoxia (data not shown). For subsequent experiments, twelve hours of hypoxia was chosen as this time approximately corresponded to the LD_{50} for both cell phenotypes.

Hypoxia induced alterations in glucose consumption and lactate production rates. Myoblasts increased glucose consumption and lactate production 3- and 2-fold respectively during hypoxia (Table I). Hypoxia induced a doubling in glucose consumption rate and a near doubling in lactate production rate in 3 day confluent cells. Although myocytes demonstrated the highest rates for glucose consumption and lactate production aerobically, this pattern was reversed by hypoxia. Hypoxia reduced myocyte glucose consumption and lactate production by 2-fold; bringing them remarkably close to the values obtained with hypoxic myoblasts.

Hypoxia-induced alterations in growth characteristics of myoblasts and myocytes. DNA histogram analysis demonstrated that hypoxia altered the cell cycle progression of myoblasts. Cells in mitosis were reduced from 25% (aerobic) to 6% (hypoxic) with a corresponding accumulation of cells in G_0/G_1 (Figure 2A and B). The percentage of S phase cells remained constant; suggesting that hypoxia blocked cells in

G_0/G_1 from entering S phase, inhibited S phase progression but allowed cells to move through G_2/M. Confluent and differentiated cells contained between 85% and 88% G_0/G_1 cells under aerobic conditions and following twelve hours of hypoxia.

Two dimensional gel analysis of proteins synthesized by aerobic and hypoxic cells. The stained two dimensional (2D) gel pattern of total cellular proteins from myoblasts and myocytes was similar, however, qualitative and quantitative differences were readily apparent (Figures 4 and 5 and ref. 16). The effect of hypoxia on protein synthesis was evaluated by comparing 2D gel patterns of two hour in situ pulse labeled proteins. In comparing the autoradiographs of myoblasts and myocytes, it became apparent that hypoxia induced an enhancement in synthesis of the 100 and 80 kDa proteins previously characterized as either glucose regulated or oxygen regulated proteins (GRP and ORP respectively, ref. 30,34). Enhanced synthesis of ORP 150 and induced synthesis of ORP 260 and 33 were also observed in both types of cells (Figures 4 and 5, Tables II and III). The differentiation state-independent induction of ORPs and GRPs in myogenic cells demonstrates the generic nature of this stress response.

A differentiation state-independent sensitivity to hypoxia was also seen in the enhanced synthesis of proteins with kDa/pI of 72/5.5 and 31/6.7 and the inhibition of alpha actin and an 86/5.6 kDa/pI protein synthesis (Figure 4 and 5, Tables II and III). Alpha actin expression is differentiation-specific however, low level expression in proliferating cells is probably due to partial up-regulation of the gene through the inevitable contact inhibition which initiates differentiation processes (39). The identity or biological function of the newly identified ORPs 72/5.5, 86/5.6 and 31/6.7 kDa/pI proteins has not been determined.

With the exception of the proteins described above, myoblast and myocytes differed markedly in which proteins were induced or inhibited by hypoxia. Considering major proteins only, synthesis of 23 proteins was altered in hypoxic myoblasts in addition to the classic five GRP/ORP (Figure 4 and Table II). Seventy percent of the alterations in protein synthesis were inhibitions. Most of these proteins have not been characterized but Western blotting with specific monoclonal antibodies demonstrated that among those proteins inhibited were the two isoforms of alpha tubulin. The two isoforms of beta tubulin were among the few proteins in proliferating cells which showed enhanced synthesis following hypoxia. A protein of 100 kDa and with apparently four isoforms, showed reduction of the two most acidic isoforms (PSP100a and PSP100b) by hypoxia. These proteins have been designated as proliferation specific proteins (PSP) because they are only detectable in proliferating myogenic cells (compare Figure 4A and B with Figure 5A and B).

Myocytes demonstrated several unique responses to hypoxia in addition to the differentiation state-independent alteration in protein synthesis. Synthesis of all the tubulin isoforms was insensitive to hypoxia but synthesis of all the actin isoforms was inhibited under hypoxic conditions (Figure 5C and D and Table III). In marked contrast to myoblasts, 75% of the alterations exclusive of the five classic GRP/ORP, involved either enhanced or induced protein synthesis (compare Table II with Table III). Most of the inductions and enhancements involved proteins of unknown biological function. The induction of the two most basic isoforms of PSP100 (PSP100c and PSP100d) and a protein with kDa/pI of 9/6.5 may have significance in terms of myogenic modulation. These proteins were synthesized in myoblasts under aerobic and hypoxic conditions, but only synthesized in differentiated cells after hypoxic stress. Inductions involving proteins 33/6.3, 32/6.3, 30/5.9, 29/6.3 may also fall into the category of PSP, however, this can not be said with certainty due to their low level of expression and the tight formation of 2D gels spots in this region (compare figure 5D with 4D).

Hypoxia potentiates the proliferative response in myocytes. The foregoing data suggested that hypoxia had modulated myocyte glucose metabolism and the synthesis of certain proteins to that characteristic of myoblasts. The most pronounced phenotypic differences between the two cellular states is that myoblasts undergo cell division, whereas myocytes do not. The effect of hypoxia on the cell cycle distribution of myocytes was examined by comparing DNA histogram of myoblasts and myocytes following replacement of the consumed media and reoxygenation. Hypoxic myoblasts demonstrated S and G2/M phase redistribution within 12 h or reoxygenation (Table IV) and continued to accumulate G2/M phase cells through 24 and 48 h of reoxygenation. A "typical" cell cycle distribution was not achieved in the time course of the experiment suggesting impaired entry into cell cycle or passage through G2/M.

Reoxygenation alone was not sufficient for the induction of a pronounced change in the hypoxic myocyte cell cycle redistribution (Table V). A small but reproducible progression of cells from S to G2/M became apparent by 24 h of reoxygenation. When reoxygenation was performed in the presence of fresh media containing serum (Table VI, 48 h/DMEM), hypoxic myocytes responded with a 1.8-fold increase in S phase distribution compared to reoxygenated cells without serum. Elevating contact inhibition by plating the cells at lower cell density (50% confluency) in the absence of serum (Table VI, 50%/48 h/N2) also stimulated an increase in S phase cells but the addition of serum (Table VI, 50%/48 h/DMEM) was still required to achieve an increment in G2/M phase cells. These data are in contrast to aerobic myocytes which do not respond to the addition of serum. In these cells a significant redistribution into S phase and G2/M was only observed when contact inhibition was relieved (Table VI, 50%/48 h/N2) and serum was added to the media (Table VI, 50%/48 h/DMEM).

In all the treatment groups described above, hypoxic cell media was replaced with fresh high glucose-containing and pH balanced media at the onset of reoxygenation. The absence of a prominant cell cycle redistribution under these conditions suggest that glucose and oxygen replenishment alone are not sufficient to elicite a full response from hypoxic myocytes. However, neither confluent or subconfluent hypoxic myocytes could respond to serum in the absence of glucose (Table VI). These data suggest that glucose is necessary but not sufficient to stimulated growth in hypoxia-modulated myocytes.

DISCUSSION

Two important questions in the areas of cancer, cardiovascular and cell trauma research are, what factors in the ischemic tissue microenvironment stress cells and how do the cells respond to these factors. These questions are of central importance in defining tumor microenvironments and their effect on cell heterogeneity in growth rate and therapeutic response (3,19-28). Oxyhemoglobin and morphometric measurements within solid tumors suggest that the cells of the microvasculature are hypoxic and show regions of disorganization (19,20). These findings suggest that microvasculature insufficiency in sold tumors may arise from factors affecting endothelial and medial cell integrity as well as from the rapid rate of tumor cell growth.

Ischemic stress also appears to be a factor in medial cell regeneration during atherogenesis (8-10,45). Several chemical and hormonal factors can stimulate regeneration of the medial cell but several studies suggest that ischemia, and in particular hypoxia, potentiates the proliferative response (8-10). In addition, atherosclerotic lesions frequently develop under these conditions at biforcations in the vessels or vessel surfaces subject to

physical or hydrostatic stress (46-48). These findings suggest ischemia predisposes the vasculature to growth which might be stimulated by growth factors and/or tissue wounding.

Ischemic tissue stress is also a significant problem in organ transplantation and recovery from severe tissue trauma, and is thought to be responsible for the phenomenum of reperfusion injury. The loss of cell viability is an important component of the compromised tissue state leading to reperfusion injury. Few studies have focused on the effects of ischemia on the vasculature of these tissues. The data presented here suggest that modulation of the differentiated cell phenotype can lead to cell proliferation which may act together with cell death to compromises the syncytium of cells comprising the vascular tissue.

The data also demonstrate that myogenic cells are sensitive to hypoxia regardless of their state of differentiation. Synthesis of the five major GRP/ORP proteins 260, 150, 100, 80, and 33 kDa were induced in both myoblasts and myocytes. Three new ORPs were identified whose synthesis was enhanced (p72/5.5 and p31/6.7) and inhibited (p86/5.6) by hypoxia in a differentiation state-independent manner. These findings suggest that proliferating cells (tumors for example) are not unique in their sensitivity to hypoxia. The effects of ischemia on differentiated non-tumor tissues are poorly understood but the data presented here suggest that part of their response to this stress will be a generic regulation of GRP/ORP synthesis.

In addition to these generic responses to ischemic stress, four responses were measured which suggest cell phenotype-specific effects. First, myocytes were more sensitive than myoblasts to short durations of hypoxia. Myoblasts on the other hand, were more sensitive than myocytes to long durations of hypoxia. The mechanistic basis for these differences are not clear. One factor which might be involved is the threshold level of oxygen which is necessary to induce either cell death or a modulation of metabolism. Thresholds could be regulated by both intracellular and extracellular factors. Alternatively, the differences in threshold sensitivity might arise from heterogeneity in the myocyte population. Differentiation involves commitment of cells to a differentiation program and entry of cells into a Go state. The myocytes were at least 90% differentiated based on morphological criteria but their metabolic characteristics and importantly, their extent of progression into the Go state might not have been homogeneous. In this regard, the Go state might be composed of several levels which differ qualititatively in cellular responsiveness to growth and stress factors. These uncertainties unscore the decision to conduct the analyses described here at a duration of hypoxia were both phenotypes had similar levels of sensitivity.

The second difference in response to hypoxia was that myoblasts demonstrated a Pasteur effect, whereas myocytes demonstrated a reverse-Pasteur effect. As myoblast differentiated into myocytes the aerobic rate of glucose consumption and lactate production increased. The simplest explanation for the apparent absence of Pasteur effect is that half of the myocyte population had been killed by hypoxia leaving the remaining cells as the sole contributers to glucose consumption and lactate production rates (hypoxic myocyte rates are approximately half that of the aerobic cells, Table I). Assuming half the number of myocytes survive hypoxia and hence half the rates of aerobic glucose consumption and lactate production (0.29 and 0.6 respectively); then hypoxic surviving myocytes would have Pasteur effects involving only 1.1-fold increases in both the glucose consumption and lactate production rates (0.33/0.29 and 0.66/0.6 respectively). Therefore despite the correction for cell number, myocytes did not demonstrate a significant Pasteur effect compare to myoblasts (3-fold increase in glucose consumption and 18-fold increase in

lactate production). These data suggest that the effect of hypoxia on metabolic rate is differentiation-dependent and that down-regulation of glucose consumption and lactate production rates is part of the hypoxic modulation of myocytes.

Myocytes also showed a third difference which involved a general up-regulation of protein synthesis. Induction of select proteins by glucose and oxygen deprivation have been well documented but these studies have been conducted on proliferating and non-differentiating cell lines (33-40). The data presented here are the first evidence of the generic nature of the GRP and ORP response as it occurs in differentiated cells. The data also demonstrate that the general qualitative nature of the response to hypoxia was not the same in myoblasts and myocytes. The bulk (70%) of hypoxia-induced alterations involving non-GRP/ORP protein synthesis in myoblasts were inhibitions. In contrast, 75% of the alterations involving non-GRP/ORP protein synthesis were either enhancements or inductions. At one level these data suggest that hypoxia down-regulates myoblast but up-regulates myocytes. This interpretation is supported by the finding that proteins which otherwise are only found in myoblasts (PSP 100 and PSP 9), are induced in myocytes by hypoxia. Induction of these proteins further supports the hypothesis that hypoxia can modulate the phenotype of myocytes with proliferative characteristics. Efforts are underway to identify these proteins by microsequencing techniques.

Though many of the proteins whose synthesis was affected by hypoxia have not been characterized, Western blotting enabled the identification of actin and tubulin isoforms. Alpha actin synthesis is a differentiated cell phenotype but occurs to some extent in myoblasts in regions where contact inhibition has taken place. Alpha actin synthesis was inhibited by hypoxia in both cell populations. The persistent dense Coomassie blue staining of actins demonstrated that synthesis and not stability of these proteins was primarily responsible for the reduced methionine labeling. Myocytes differed from myoblasts in that the de novo synthesis of alpha, beta and gamma actins were inhibited by hypoxia. The molecular basis for these observations were not evaluated further. Phenotype of the cells was also a discriminating factor in the sensitivity of tubulin synthesis to hypoxia. Tubulin synthesis appeared to be refractile to hypoxia in myocytes but in myoblasts, alpha tubulin synthesis was inhibited and beta tubulin was among the few proteins in these cells to show enhanced synthesis. Although these observations are descriptive, they serve to further unscore the diverse levels where differentiation can affect the responsivity of cells to environmental stimuli such as hypox stress.

The fourth and most important end point of modulation measured in this study was re-entry of myocytes into the cell cycle. Reoxygenation of hypoxic myoblasts demonstrated their capability to promptly resume S phase progression through G2/M. The cell cycle distribution was however atypical in that cells appeared to be able to progress through S phase and perhaps enter S phase from G1 (as indicated by the rise in G2/M phase distribution by 48 h of reoxygenation) but they appeared to accumulate in G2/M suggesting an impaired ability to progress through mitosis. These cells eventually (4-6 days) acquire a normal cell cycle distribution. The transient impairment to G2/M progression might be related to the hypoxia-induced aberrant synthesis of alpha and beta tubulins.

Myocytes were predominantly in G1/G0 and this distribution was not altered by 12 h of hypoxia. Reoxygenation induced a modest but reproducible redistribution of cells. The possibility that the hypoxia had modulated these cells became apparent when growth factors were supplied during the reoxygenation period. Under these conditions the S and G2/M phase populations increased 1.8- and 1.4-fold respectively, compared to serum-treated aerobic myocytes. The potentiation of a proliferative response in myocytes by

hypoxia was apparent in the lack of responsiveness of aerobic myocytes to serum and their dependency on having contact inhibition relieved (replating at subconfluent cell density) before serum responsiveness could be demonstrated. Release from contact inhibition slightly increased the ability of hypoxic cells to respond to serum growth factors, but apparently was not an absolute requirement. An intriguing possibility is that loss of contact inhibition might be related to hypoxia-induced alterations in actin synthesis.

Taken together, the data suggest that hypoxia can modulate differentiated myogenic cells with proliferative characteristics, including altered glucose metabolism, protein synthesis and responsiveness to growth factors. These data have important implications as they suggest that ischemia can traumatize vascular beds by: 1) killing cells and 2) modulating differentiated cells to proliferate and thereby compromising the syncytium of cells comprising vessel walls. The data also suggest that ischemia will have a different effect on and prognosis for tissues with high mitotic index compared to differentiated tissues.

MATERIALS AND METHODS

Cell Growth Conditions. BC_3H1 cells were maintained as a proliferating stock on plastic T-flasks in Dulbecco's Modified Eagles Medium (DMEM) containing 4 mg/ml glucose and 10% fetal calf serum (Gibco, Grand Island, NY) and grown in the presence of 5% CO_2. Cells were maintained at 40-60% confluency by periodic trypsinization and replating. Alternatively, proliferating stocks were immobilized as 2 mm Ca^{+2}-alginate beads (Bellco Glass Inc., Vineland, NJ) according to the manufactuer's instructions and grown to mid log phase in DMEM containing 10% fetal calf serum using a 1-3 liter continuous perfusion airlift bioreactor (Bellco Glass Inc.).

BC_3H1 cells were differentiated as described previously (5). Briefly, once cells became 60% confluent, the spent media was replaced with fresh DMEM and the cells were cultured without additional changes of media until the cells had been confluent for a minimum of three days. Confluency and the cessation of growth were evaluated flow cytometrically (see below). At this point, the spent media was replaced with serum-free N_2 media [RPMI 1640 (Gibco) 5 μM fatty acid free bovine serum albumin (Miles Inc. Kankakee, IL), 5 μg insulin/ml (Miles Inc.), 100 μg transferrin/ml (Sigma, St. Louis, MO), 100 μM putrescine (Sigma), 30 nM Na_2SeO_3, 20 nM progesterone (Sigma) and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, pH=7.2] and the cultures were maintained for four-five days without replacing the media at which point 80-90% of the culture appeared morphologically differentiated (Figure 1B).

Establishing Hypoxic Cultures. Cells grown as described above were given fresh DMEM or N_2 media and immediately placed in evacuation chambers (7). The chambers were intermittently degassed under vacuum and flushed with 5% $C0_2$/95% N_2 at room temperature for approximately one hour and then stored at 37°C for the indicated times. Under these conditions, oxygen levels drop to 10 mm by 8-12 h of hypoxia and cells become radiobiologically hypoxic (1,7,9,10). Alternatively, hypoxic cultures were established and maintained as bioreactor cultures using a microprocessor PID controller unit, an in-culture oxygen sensor probe and compressed air and 5% CO_2/95% N_2 for regulating dissolved oxygen at 2100 ppm.

Glucose and Lactate Analyses. Glucose and lactate concentrations were determined enzymatically using commercial glucose oxidase and lactate dehydrogenase assay kits (Sigma). Glucose and lactate were assayed from a common aliquot of spent media which had been exposed to cells for a fixed period (12 hours). Fresh DMEM and N_2 media

served as the T_0 glucose consumption and lactate production control. The rate of glucose consumption was calculated as the difference between the glucose concentration in the media at T_0 and T_{12} divided by 12 hours and corrected for cell number. The rate of lactate production was calculated as the difference between the lactate concentration in the media at T_{12} and T_0 divided by 12 hours and corrected for cell number.

Cell Survival Analysis. Following the indicated periods of hypoxia, hypoxic cells were trypsinized from plates or dissolved out of alignate with 10 m EGTA and trypsinized and replated at 100-, 500- and 1000-fold dilution and allowed to grow for 2-3 weeks in DMEM media containing 10% fetal calf serum. Plating dilutions were performed in triplicate. Colonies were stained with a 1% solution of crystal violet and counted manually. Aerobic cultures which had been established as part of the same batch as those which were made hypoxic, were treated in a similar fashion and served as a plating efficiency control.

Cell Cycle Analysis. Aerobic and hypoxic cells at the various stages of differentiation indicated, were fixed in 70% ethanol (-20°C) and kept at -20°C until fluorescence activated cell sorting (FACS) analysis. Fixed cells were then centrifuged (500 X g, 10 min), resuspended in 2 ml of PBS containing 50 μg RNase A/ml and incubated at room temperature for 30 min. Propidium iodide was added (2 mg/ml final concentration) and FACS performed on and Epics Profile flow cytometer (Coulter Counter, Hialeah, FL).

Radiolabeling Total Cellular Proteins <u>In situ</u>. Aerobic and hypoxic cells were rinsed with methionine-free media (DMEM based for proliferating cells and RPMI-1640 based for differentiated cells) and incubated aerobically at 37°C for 2 hours in 50 mls of methione-free media containing either 10% dialyzed fetal calf serum (Gibco) or N_2 components (proliferating and differentiated cells respectively) and 50 μCi of [^{35}S]-methionine (Amersham, Boston, MA). Aerobic conditions were used to radiolabel both aerobic and hypoxic cells as it improved the efficacy of isotope uptake by hypoxic cells (19,34).

Preparation of Total Cellular Proteins for Two Dimensional Gel (2D P.A.G.E.) Analysis. Following pulse labeling, cells were washed twice with fresh DMEM (without serum) (800 X g, 2 min), resuspended in 20 volumes of S.T.O.P. buffer (50 mM Tris (pH=8.0), 5 μg/ml of leupeptin (Japanese Peptide Inst.), aprotinin (Sigma) soybean trypsin inhibitor (Sigma) and 1 mM phenyl methyl sulfonyl fluoride, PMSF (Sigma) and incubated on ice for 30 min. The swollen cells were sheared by sequential passage through 18, 22 and 26 gauge needles and protein concentration determined using the BioRad assay (Bio Rad Lab, Rockville Centre, NY). Cellular material was made 5 mM $MgCl_2$, 5 μg DNase I (Sigma) and 5 μg RNase A (Sigma)/mg total protein and digested at 37°C for 30 min. Following nucleic acid digestion, proteins were precipitated with 5 volumes of -20°C acetone and incubated on ice for 30 min followed by centrifugation at 5000 X g, 20 min. Protein pellets were briefly air dried, resuspended to 2 mg protein/ml in isoelectric focusing (I.E.F.) sample buffer (9M urea, 2 mM dithiothreitol, 2% ampholine (3-10 range, BioRad) and 0.4% NP-40 and following a brief incubation at 50°C and vortexing, insoluble material was cleared by centrifugation (100,000 X g, 30 sec).

2D P.A.G.E. Analysis and Autoradiography. A standard amount of total cellular proteins (200 μg containing 80,000 dpms) were polymerized in 4% polyacrylamide tube gels containing 3-10 ampholines and isoelectric focusing (I.E.F.) electrophoresis carried out to equilibrium (14 hours) as described by O'Farrell (43). The pH gradient was determined on 5 mm thick slices from gels run in parallel.

I.E.F. gels were conditioned with sodium dodecyl sulfate (SDS) treatment buffer (62.5 mM Tris pH-6.8, 10% glycerol, 2% 2-mercaptoethanol, 1% SDS, 0.05% Bromophenol blue)

for 20 min at room temperature and sealed onto the 4.5% polyacrylamide stacking gel of the second dimensional gel with 0.5% agarose. The molecular weight resolving portion of the second dimensional gels was a 5-18% polyacrylamide gradient SDS gel (44). Following electrophoresis, gels were fixed overnight in 10% acetic acid and 40% methanol, stained with Coomassie Blue, destained, dried between plastic and exposed to XRP-5 X-ray film for varying durations. Autoradiographs resulting from similar exposures were selected for photography and manual analysis.

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FOOTNOTES

[1]Roll, E.E., Murphy, B.J., Laderoute, K.R., Sutherland, R.M. and Smith, H.C. Oxygen regulated 80 kDa protein and glucose regulated 78 kDa protein are identical. Manuscript submitted.

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TABLE I Alterations in Glucose Consumption and Lactate Production Rates

CELLULAR STATE[1]	OXYGEN[2]	MEDIUM	GLUCOSE (mM)[3]	LACTATE (mM)[3]
———	Aerobic	DMEM	22	1.7
Myoblasts	Aerobic	DMEM	21 (0.08)[4]	6.0 (0.36)[4]
Myoblasts	Hypoxic	DMEM	19 (0.25)	9.2 (0.63)
3 day Confluent	Aerobic	DMEM	18 (0.33)	8.0 (0.53)
3 day Confluent	Hypoxic	DMEM	13 (0.75)	12 (0.86)
———	Aerobic	N2	23	BD
Myocytes	Aerobic	N2	16 (0.58)	14 (1.2)
Myocytes	Hypoxic	N2	19 (0.33)	8.1 (0.66)

[1] Cells were grown under the conditions described in Materials and Methods and monitored by microscopy as shown in Figure 1. All values represent the average of three separate determinations. S.E.M. is ± 2 mM and ± 3 for glucose and lactate determinations respectively.

[2] Cells were grown aerobically with 5% CO_2 or made hypoxic as described in Materials and Methods.

[3] Glucose and lactate were measured directly in the media using the enzymatic assays described in Materials and Methods after 12 hours of exposure to the culture.

[4] *Numbers within parentheses are the rates of consumption or production (for glucose and* lactate respectively) in mM/hr and corrected for cell number as described in Materials and Methods.

TABLE II Abundant Proteins in Myoblasts Whose Synthesis is Affected by Hypoxia

DESIGNATION[1]	Mr/pI	EFFECT OF HYPOXIA[2]
ORP	260/5.2	▲
ORP	150/5.3	▲
PSP	100/6.4	▼
PSP	100/6.5	▼
GRP	100/4.9	▲
—	86/5.6	▼
GRP	80/5.1	▲
—	72/5.5	▲
—	69/5.4	▼
α Tubulin	60/6.7	▼
α Tubulin	60/6.8	▼
β Tubulin	60/6.9	▲
β Tubulin	60/7.0	▲
—	48/5.8	▲
—	47/6.3	▲
α actin	45/5.2	▼
—	41/6.3	▲
—	37/6.6	▼
—	36/6.7	▼
ORP	33/7.0	▲
—	31/6.7	▲
—	24/8.1	▼
—	21/6.5	▼
—	15/5.0	▼
—	11/5.3	▼
—	10/6.5	▼
—	9/6.3	▼
—	8/6.6	▼

[1] The designation for the proteins was given based on the known Mr/pI coordinates of the oxygen regulated proteins (ORPs) or in the case of tubulin and actin, by Western blotting of 2D gels with antibodies specific for these proteins.

[2] Upward arrowheads indicate enhanced or induced synthesis and downward arrowheads indicate reduced synthesis. Effects were evaluated by direct visual comparison of Figure 4 to Figure 5.

TABLE III Abundant Proteins in Myocytes Whose Synthesis is Affected by Hypoxia

DESIGNATION[1]	Mr/pI	EFFECT OF HYPOXIA[2]
ORP	260/5.2	▲
ORP	150/5.3	▲
PSP	100/6.6	▲
PSP	100/6.7	▲
GRP	100/4.9	▲
—	86/5.6	▼
GRP	80/5.1	▲
—	72/5.5	▲
—	52/8.2	▲
—	48/5.8	▲
—	47/7.4	▲
—	47/6.3	▲
α actin	45/5.2	▼
β actin	45/5.3	▼
γ actin	45/5.5	▼
—	40.4/6	▲
—	38/4.9	▼
—	36/7.9	▲
ORP	33/7.0	▲
PSP?	33/6.3	▲
PSP?	32/6/3	▲
PSP?	30/5.9	▲
PSP?	29/6.3	▲
—	25/4.7	▲
PSP	9/6.5	▲

[1] The designation for the proteins was given based on the known Mr/pI coordinates of the oxygen regulated proteins (ORPs) or in the case of tubulin and actin, by Western blotting of 2D gels with antibodies specific for these proteins.

[2] Upward arrowheads indicate enhanced or induced synthesis and downward arrowheads indicate reduced synthesis. Effects were evaluated by direct visual comparison of Figure 4 to Figure 5.

TABLE IV. CELL CYCLE DISTRIBUTION OF HYPOXIC MYOBLASTS FOLLOWING REOXYGENATION

Group[1]	Reoxygenation[2] Conditions	S Phase[3]	G2/M Phase[3]
Aerob	-	35%	25%
Hypox	-	35%	6%
Hypox	12 h/DMEM	16%	25%
Hypox	24 h/DMEM	16%	41%
Hypox	48 h/DMEM	17%	35%

[1] Myoblasts (40-60% confluent proliferating cells) were grown aerobically (Aerob) or at 2100 ppm oxygen (Hypox) as described in Methods.

[2] Following 12 h of hypoxia, the media was replaced with fresh DMEM (proliferation media) for 12-48 h of reoxygenation.

[3] The cell cycle distribution was determined with software provided by Coulter with the "Epics Profile" flow cytometer.

TABLE V. CELL CYCLE DISTRIBUTION OF HYPOXIC MYOCYTES FOLLOWING REOXYGENATION

Group[1]	Reoxygenation[2] Conditions	S Phase[3]	G2/M Phase[3]
Aerob	-	15%	5%
Hypox	-	14%	6%
Hypox	24 h/N2	10%	12%
Hypox	48 h/N2	11%	12%

[1] Myocytes (differentiated myogenic cells) were grown aerobically (Aerob) or at 2100 ppm oxygen (Hypox) as described in Methods.

[2] Following 12 h of hypoxia, the media was replaced with fresh N2 media (differentiation media) for 24-48 h of reoxygenation.

[3] The cell cycle distribution was determined with software provided by Coulter with the "Epics Profile" flow cytometer.

TABLE VI. POTENTIATION OF THE PROLIFERATIVE PHENOTYPE IN MYOCYTES BY HYPOXIA

Group[1]	Reoxygenation[2] Conditions	S Phase[3]	G2/M Phase[3]
Hypox	48 h/DMEM	21%	13%
Hypox	50%/48 h/N2	23%	5%
Hypox	50%/48 h/DMEM	23%	14%
Aerob	48 h/DMEM	12%	9%
Aerob	50%/48 h/N2	19%	1%
Aerob	50%/48 h/DMEM	20%	11%
Hypox	48 h/N2 (+) Serum (-) Glucose	14%	8%
Hypox	50%/48 h/N2 (+) Serum (-) Glucose	15%	5%

[1] Myocytes (differentiated myogenic cells) were grown aerobically (aerob) or at 2100 ppm oxygen (Hypox) as described in Methods.

[2] Following 12 h of hypoxia, the media was replaced with fresh N2 media (differentiation media), DMEM media (proliferation media) of N2 media (minus glucose) supplemented with 10% dialyzed fetal calf serum (N2/+serum/-glucose). Alternatively, cells were plated at subconfluent density (50%) in these three media.

[3] The cell cycle distribution was determined with software provided by Coulter with the "Epics Profile" flow cytometer.

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Figure 1

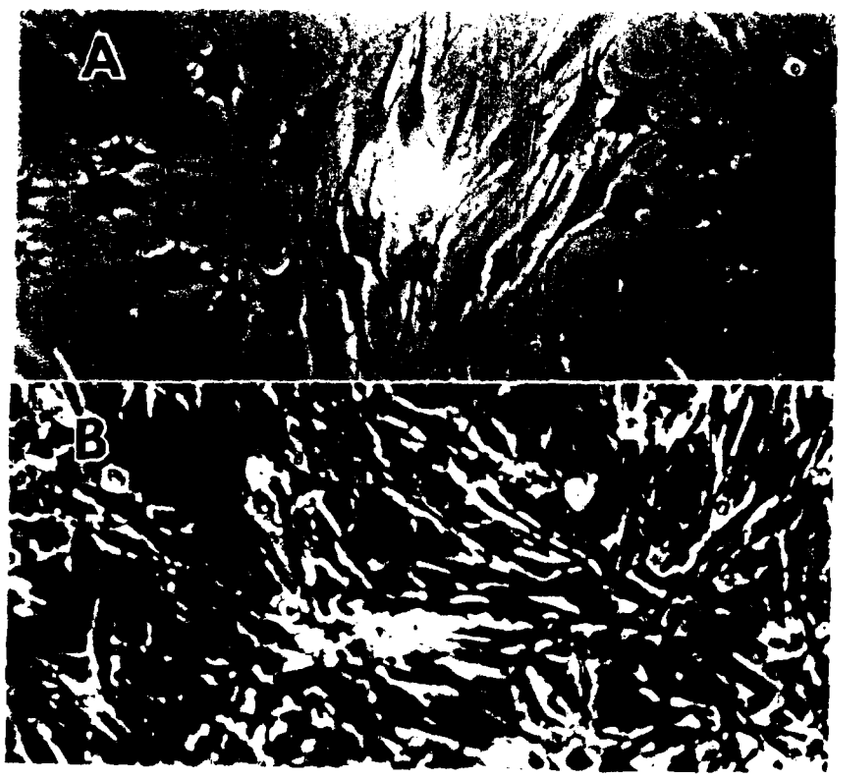


Figure 2

Cell Survival After Hypoxia

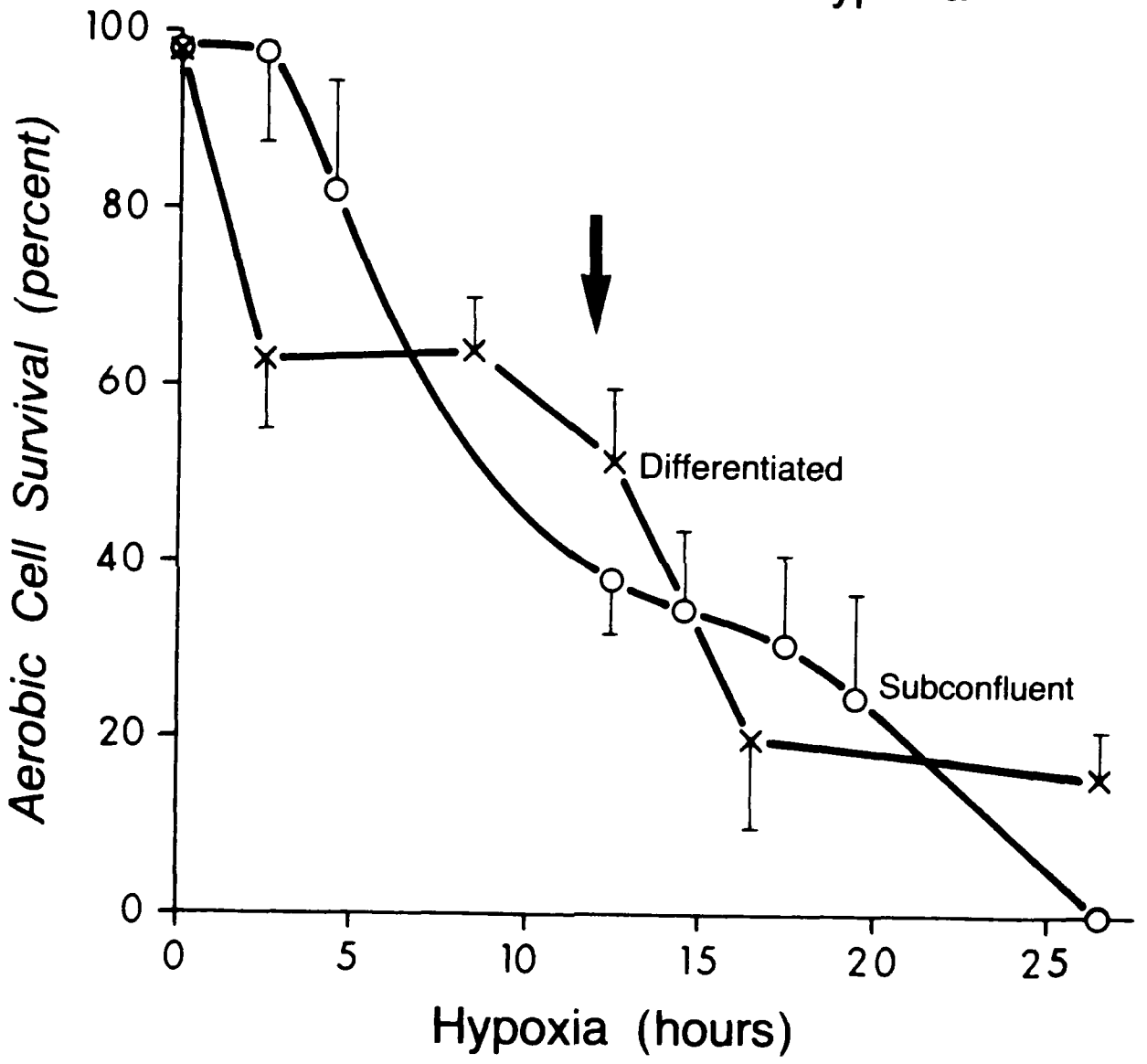
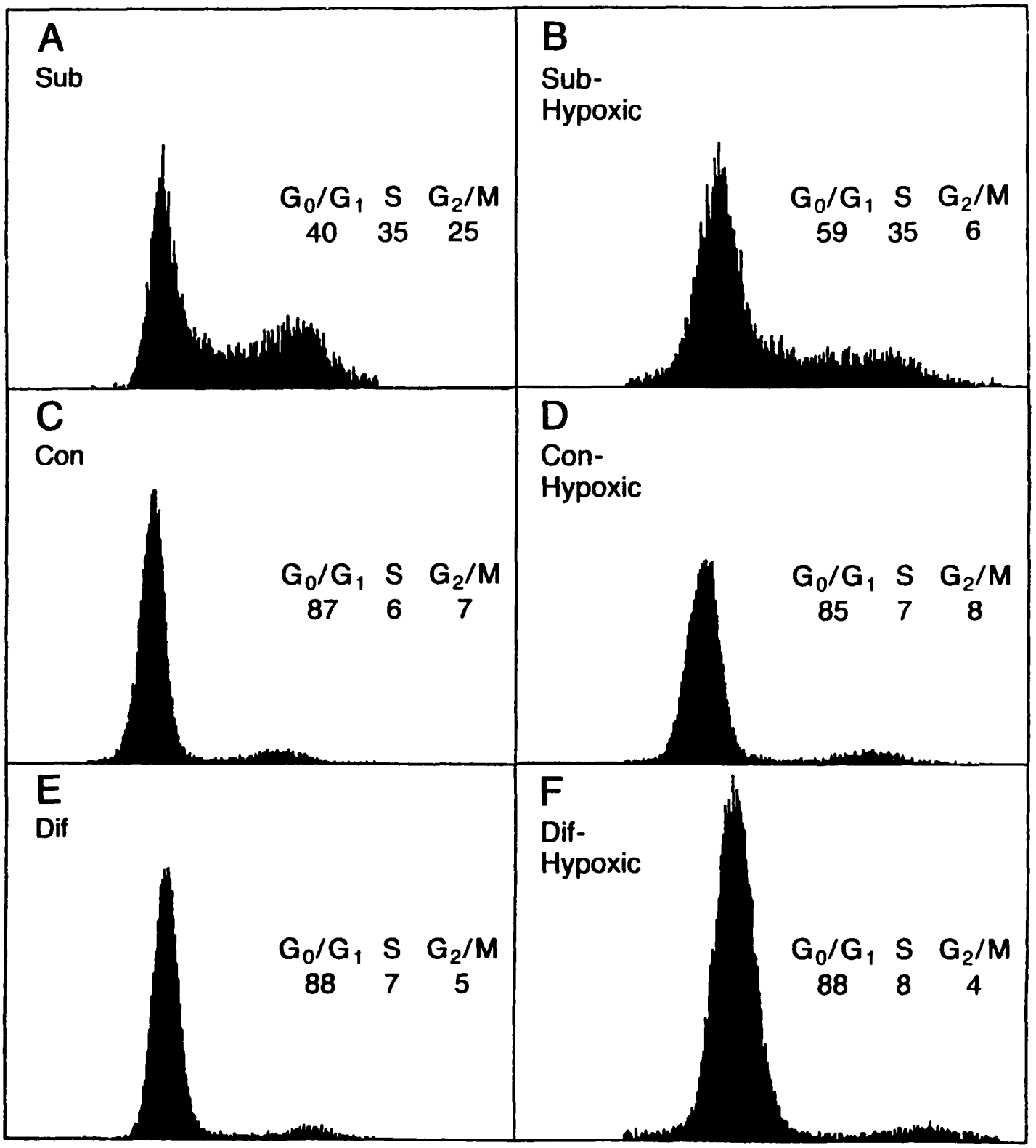


Figure 3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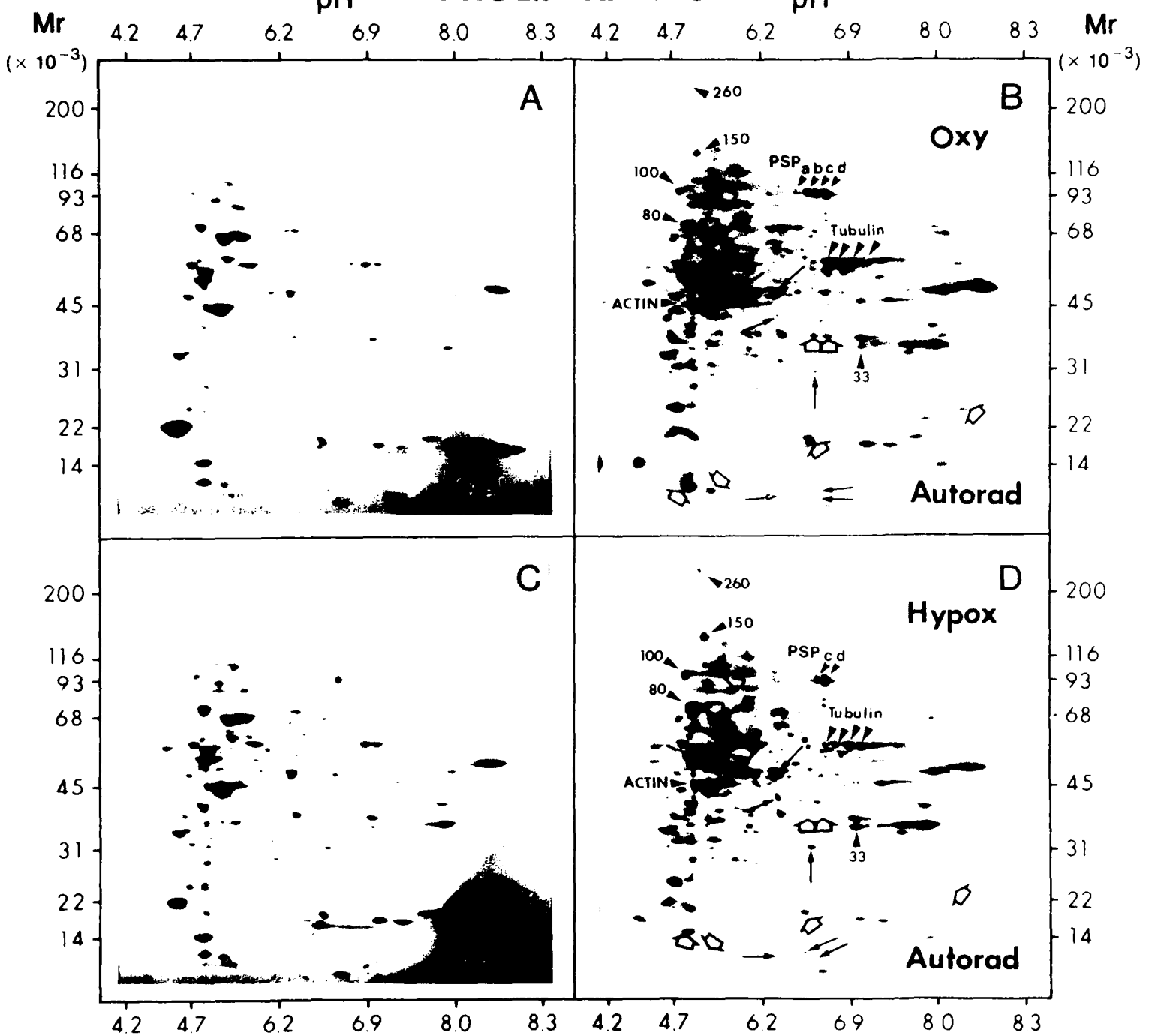
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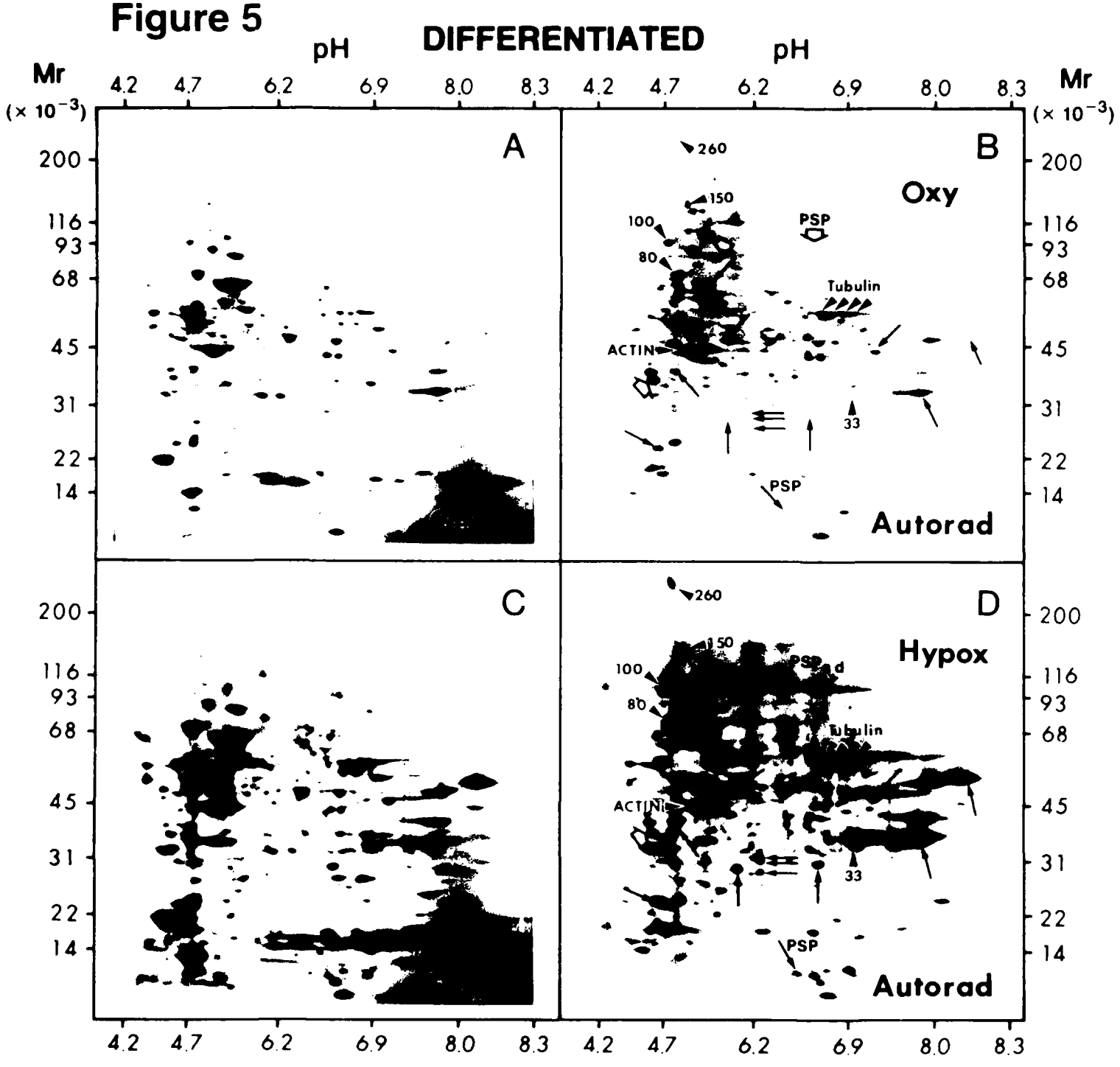
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